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The antitumor sulfonylurea *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea (LY181984) inhibits NADH oxidase activity of HeLa plasma membranes

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Abstract

Plasma membrane vesicles from HeLa S cells grown in culture bound with high affinity the antitumor sulfonylurea *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea (LY181984). Based on binding site protection experiments with the radiolabeled thiol reagent *N*-[^{14}C]ethylmaleimide, a ca. 34 kDa binding protein was identified. By analogy with a 36 kDa NADH oxidase from plant plasma membranes where activity was blocked by a growth-inhibitory herbicidal sulfonylurea, the sulfonylurea-binding protein of the HeLa plasma membranes has now been identified as a comparable sulfonylurea-inhibited NADH oxidase activity. The drug inhibited half maximally at about 50 nM which corresponded closely to the K_d for binding of [^{3}H]LY181984 of 25 nM. A closely related but growth-inactive sulfonylurea *N*-(4-methylphenylsulfonyl)-*N'*-(phenyl)urea (LY181985) inhibited the activity only weakly. The inhibition by LY181984 was analyzed kinetically and shown to be noncompetitive or uncompetitive depending on the concentration of NADH. With sealed right-side out plasma membrane vesicles, the NADH oxidase activity was about 90% inhibited by 1 μM LY181984. With frozen and thawed plasma membrane vesicles or with vesicles first solubilized with 1% Triton X-100, activity also was inhibited by LY181984 and not by LY181985 but the maximum inhibition at 10 μM LY181984 was about 50%. With plasma membranes from rat liver, neither LY181984 nor LY181985 affected the NADH oxidase even in the presence of detergent. Thus, selective inhibition or stimulation of the oxidation of NADH of tumor plasma membranes by the antitumor sulfonylurea LY181984 may be related to its antitumor activity.

Keywords: Diarylsulfonylurea; Sulfonylurea; Antitumor drug; NADH oxidase; Plasma membrane; HeLa cell; Hepatoma; (Rat liver)

1. Introduction

Homogenates, total particulate and plasma membranes of cultured HeLa S cells bound the tritiated antitumor sulfonylurea [^{3}H]LY181984 (*N*-(4-methylphenyl)-*N'*-(4-chlorophenyl)urea) with high affinity (K_d of 20 to 50 nM) [1]. Highest affinity binding was to purified plasma membranes (K_d of 25 nM). In a subsequent report [2], a binding protein, apparently with one or more thiols in the active site, was identified and labeled with radioactive thiol reagents in sulfonylurea protection experiments. Binding proteins of M_r ca. 34 kDa were labeled [2].

In this report, we provide evidence that the 34 kDa sulfonylurea-binding protein has NADH oxidase activity. The experimental approach was based on earlier findings in plants of an NADH oxidase activity that responded to auxin regulators of plant growth [3]. This activity was not only stimulated by the auxin regulators but was inhibited as well by herbicidal sulfonylureas [4] that at subherbicidal concentrations may act as plant growth retardants [5]. As a result, the comparable cyanide-resistant oxidation of NADH by plasma membranes of HeLa cells was tested for inhibition by the antitumor sulfonylurea LY181984. LY181984, but not a structurally-related but antitumor-inactive sulfonylurea, *N*-(4-methylphenylsulfonyl)-*N'*-(phenyl)urea (LY181985), inhibited the activity. By kinetic analysis, the inhibition was shown to be uncompetitive or noncompetitive depending on NADH concentration. Additionally, the comparable cyanide-resistant NADH oxida-

Abbreviations: LY181984, *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea; LY181985, *N*-(4-methylphenylsulfonyl)-*N'*-(phenyl)urea; Sulofenur, LY186641, *N*-(5-indanylsulfonyl)-*N'*-(4-chlorophenyl)urea.

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tion by liver plasma membranes was not affected by either LY181984 or LY181985 in keeping with the antitumor specificity of the sulfonylureas.

Taken together, the findings suggest that the 34 kDa sulfonylurea-binding protein of HeLa plasma membranes may be the transformed cell equivalent of the auxin-responsive NADH oxidase of plant membranes. The drug responsiveness of the HeLa activity provides an important difference between this activity and that of a growth factor- and hormone-stimulated NADH oxidase of liver plasma membranes also resistant to cyanide [6,7]. While the growth factor- and hormone-responsiveness of the plasma membrane NADH oxidase appeared to be lost upon transformation [8,9], the response to sulfonylurea provides additional evidence of the existence in mammalian plasma membranes of an NADH oxidase activity altered in transformation.

The sulfonylureas represent a novel series of synthetic organic compounds identified as having activity against human solid tumors in vivo [10–12]. Known collectively as antitumor diarylsulfonylureas (sulfonylureas), the compounds have a high degree of efficacy and a relatively low toxicity [10,12]. Additionally, their mechanism of action, while unknown, is apparently unrelated to previously described classes of oncolytic agents [13]. One member of the series, Sulofenur, progressed in evaluation to Phase I [12,13] and Phase II [14] clinical trials. The sulfonylureas were identified as the result of a program of screening against in vivo murine solid tumors implanted subcutaneously [11].

Despite considerable clinical and laboratory data, the site of antitumor sulfonylurea action has remained elusive [10,15]. The drugs are membrane active and weak uncouplers of mitochondrial oxidative phosphorylation [15–18]. There is no evidence for cell cycle specificity of the drugs and no inhibition of DNA, RNA or protein synthesis [13,15]. The sulfonylureas exhibit few, if any, mechanistic parallels to other known antitumor agents [19]. Thus, their mode of action is expected to be unique.

2. Materials and methods

2.1. Growth of cells

HeLa S cells were grown on minimal essential medium (S-MEM) (Jolik modified) with glutamine (244 mg/l) and phosphate (1.3 g/l Na_2HPO_4) and without $CaCl_2$ plus 5% donor horse serum. Gentimycin sulfate (50 mg/l) and sodium bicarbonate (2 g/l) were added. Cells were collected by centrifugation for 6 to 15 min at 1000 to 3000 rpm (e.g., 6 min at 3000 rpm or 15 min at 1000 rpm).

Attached HeLa cells (ATCC CCL2), were grown in 150 cm flasks in minimal essential medium (Gibco), pH 7.4, at 37°C with 10% bovine calf serum (heat-inactivated), plus 50 mg/l gentimycin sulfate (Sigma). Cells were trypsinized with Sigma IX trypsin for 1 to 2 min and harvested by scraping and taken up in TD-Tris buffer (140 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 25 mM Tris, pH 7.4) to a final cell concentration of 0.1 g wet weight per ml. Cell survival was determined by Eosin Y exclusion.

2.2. Purification of plasma membranes from HeLa cells

HeLa cells grown as suspension cultures were collected by centrifugation for 6 to 15 min at 1000 to 3000 rpm (e.g., 6 min at 3000 rpm or 15 min at 1000 rpm). The cell pellets were resuspended in 0.2 mM EDTA in 1 mM $NaHCO_3$ in an approximate ratio of 1 ml per 10^8 cells and incubated on ice for 10 to 30 min to swell the cells. Homogenization was with a Polytron homogenizer for 30 to 40 s at 10 500 rpm using a PT-PA 3012/23 or ST-probe in 7 to 8 ml aliquots. To estimate breakage, the cells were monitored by light microscopy before and after homogenization. At least 90% cell breakage without breakage of nuclei was achieved routinely.

The homogenates were centrifuged for 10 min at 1000 rpm ($175 \times g$) to remove unbroken cells and nuclei and the supernatant was centrifuged a second time for 1 h at $23\,500 \times g$ to prepare a plasma membrane-enriched microsome fraction. The supernatant was discarded and the pellets were resuspended in 0.2 M potassium phosphate buffer in a ratio of approx. 1 ml per pellet from $5 \cdot 10^8$ cells. The resuspended membranes were then loaded onto the two-phase system constituted on a weight basis as follows. The two-phase system contained 6.6% (w/w) Dextran T-500 (Pharmacia), 6.6% (w/w) poly(ethylene glycol) 3350 (Fisher), and 5 mM potassium phosphate buffer, pH 7.2 [20]. The homogenate (1 g) was added to the two-phase system and the weight of the system was brought to 8 g with distilled water. The tubes were inverted vigorously for 40 times in the cold (4°C). The phases were separated by centrifugation at 750 rpm ($150 \times g$) in a Sorvall HB 4 rotor for 5 min. The upper phases were carefully withdrawn with a pasteur pipette, divided in half and transferred into 40 ml plastic centrifuge tubes and diluted with cold 1 mM $NaHCO_3$ and centrifuged at $10\,000 \times g$ in a HB rotor for 30 min. The purity of the plasma membrane was determined to be > 90% by electron microscope morphometry. The yield was 20 mg plasma membrane protein from 10^{10} cells.

2.3. Purification of plasma membranes from rat liver

Rat liver plasma membranes were purified from the $5000 \times g$ pellet from the preparation of Golgi apparatus [21] which was the starting material. The fluffy layer which contains the Golgi apparatus was mixed, withdrawn and excluded from the plasma membrane preparations. Cold 1 mM $NaHCO_3$ (5 ml) was added to each tube and the friable yellow-brown part of the pellet was resuspended with a pen brush, leaving the reddish, tightly-

packed bottom part of the pellet undisturbed. The resuspended material was homogenized in aliquots of 5 ml each in a 30 ml stainless steel (Duragrind) homogenizer 20 times by hand. The homogenates were combined, diluted with cold 1 mM $NaHCO_3$ (1:1 dilution), and the pellet was used for the two-phase separation as described above for HeLa cell plasma membranes.

The plasma membrane preparations from rat liver have been characterized extensively based on both morphological and enzymatic criteria [20,22]. From morphometric analysis using electron microscopy, the preparations contained 90 ± 4 percent plasma membrane. Contaminants included mitochondria (4%) and endoplasmic reticulum (3%). Based on analyses of marker enzymes, the contamination by endoplasmic reticulum was estimated to be 3%, that of mitochondria 15% and that of Golgi apparatus 1%. The yield of plasma membranes was estimated to average 18% based on recovery of marker enzymes.

Plasma membrane pellets were resuspended in 50 mM Tris-Mes buffer (pH 7.0) and stored at −70°C. Proteins were determined using the bichinchoninic acid (BCA) assay [23] with bovine serum albumin as standard.

2.4. Spectrophotometric assay

NADH oxidase activity was determined as the disappearance of NADH measured at 340 nm in a reaction mixture containing 25 mM Tris-Mes buffer (pH 7.2), 1 mM KCN to inhibit any potential mitochondrial oxidase activity, and 150 μM NADH at 37°C with constant stirring. Activity was measured using a Hitachi U3210 with continuous recording over 5 or 10 min intervals. A millimolar extinction coefficient of 6.22 was used to determine NADH disappearance.

3. Results

The NADH oxidase activity of HeLa cell plasma membranes was characterized and shown to have steady-state characteristics similar to those of rat liver (Table 1). When assayed for sulfonylurea responsiveness, the NADH oxidase of the isolated plasma membrane vesicles of HeLa cells was inhibited by the antitumor active LY181984 but not by the inactive LY181985 (Table 2, Fig. 1). With freshly isolated right side-out vesicles stored overnight at 4°C, half maximal inhibition was at about 50 nM LY181984 and inhibition was about 90% at 1 μM (Fig. 1). The inactive LY181985 was without effect until 10 μM where a slight (20%) inhibition was observed.

With plasma membranes stored frozen and then thawed, the specific activity of the NADH oxidase was approx. 1.9 that of freshly prepared vesicles but the overall degree of inhibition by the antitumor active LY181984 was only about 50% (Fig. 2). The antitumor inactive LY181985 was only slightly (about 10% at 10 μM) inhibitory.

When the response to increasing amounts of NADH was determined, the resultant kinetics were complex (Fig. 3). At high NADH, the inhibition by LY181984 appeared to be uncompetitive. Both the K_m and the V_{max} were de-

Table 1
Characteristics of NADH oxidase activity comparing rat liver, rat hepatoma and HeLa cell plasma membranes

Characteristics	Source of plasma membrane		
	liver	hepatoma	HeLa
pH optimum	7.0	7.0	7.0
K_m for NADH (μM) (0–30 μM NADH)	≈ 25	≈ 25	≈ 25
Ion requirements	none	none	none
Specific activity (nmol/min per mg protein)	1.5–2.1	1.6–2.4	0.8–1.6

Table 2
NADH oxidase activity of plasma membranes from cultured cells and rat liver

Cells or tissue	NADH oxidase (nmol/min per mg protein)		
	DMSO alone	+LY181984	+LY181985
HeLa cells	0.85 ± 0.05	0.15 ± 0.03	0.85 ± 0.05
Rat kidney cells	2.2 ± 0.09	2.2 ± 0.13	2.2 ± 0.11
Rat liver	1.75 ± 0.3	1.8 ± 0.2	1.75 ± 0.2

The sulfonylurea concentration was 1 μM. The final DMSO concentration was 0.1%.

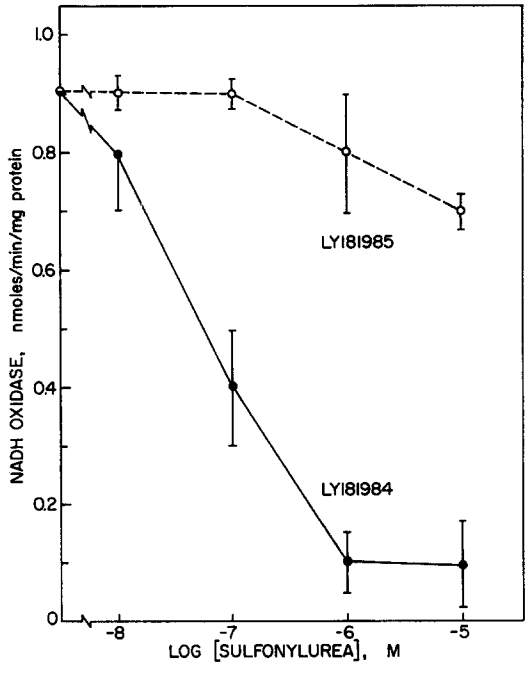

Fig. 1. Inhibition of NADH oxidase activity of HeLa plasma membranes by increasing concentrations of LY181984 (solid symbols) and LY181985 (open symbols). The antitumor active LY181984 inhibited NADH oxidase activity with sealed right-side-out vesicles. The chemically-related but antitumor inactive LY181985 required several log orders more drug to inhibit. For each experiment duplicate determinations were averaged. Results are based on averages of three experiments ± standard deviations among experiments.

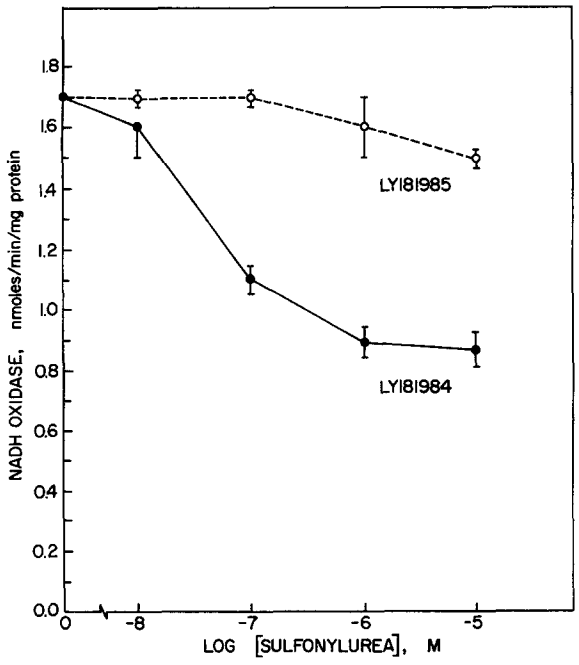

Fig. 2. With plasma membranes that were stored frozen and then thawed, the NADH oxidase activity was inhibited by LY181984 (solid symbols) but not by LY181985 (open symbols). The specific activity of the plasma membrane NADH oxidase was higher after freezing and thawing but the extent of inhibition by the antitumor active LY181984 was less that with freshly prepared, predominantly right side-out, vesicles (Fig. 1). The antitumor inactive LY181985 was only weakly inhibitory. For each experiment duplicate determinations were averaged. Results are based on averages of three experiments ± standard deviations among experiments.

creased in the presence of LY181984. However, at low NADH, the analyses approximated noncompetitive kinetics. The latter might indicate the presence of a high K_m form of the oxidase differentially affected by the sulfonylureas (Fig. 3B). With the frozen and thawed preparations averaged from 8 determinations, the apparent V_{max} was 0.5 ± 0.2 nmol NADH oxidized per min per mg protein in the presence of LY181984 whereas in the absence of LY181984 the apparent V_{max} was 1.2 ± 0.3 nmol NADH oxidized/min per mg protein.

With freshly isolated vesicles, presumably sealed and predominantly right side-out, kinetic analyses yielded primarily the low K_m form (K_m for NADH = 25–30 μM) of the activity at high NADH concentrations (Fig. 4). However, at low NADH, an activity with an apparent K_m for NADH of about 60 μM also was observed which was no longer apparent at 1 μM LY181984. The apparent V_{max} in the absence of LY181984 was 1.3 nmol/min per mg protein, whereas in its presence at 1 μM, the apparent V_{max} was 0.15 nmol/min per mg protein. Results with 0.1 μM LY181984 were intermediate.

Growth of attached HeLa cells in culture was reduced by the antitumor sulfonylurea LY181984 with an EC_{50} of about 50 μM (Fig. 5). It has generally been observed that transformed cells in culture were less responsive to the antitumor sulfonylureas than was the in vivo growth of solid tumors [10]. The antitumor inactive analog LY181985 was without effect on cell growth even at 100 μM. With plasma membranes of HeLa cells, LY181984 but not LY181985 inhibited as well in detergent-solubilized preparations (Fig. 6). Specific activity was not increased by detergent compared to frozen and thawed vesicles and the maximum inhibition by LY181984 was about 50% with the detergent-solubilized activity (Fig. 6).

Neither LY181984 nor LY181985 affected the NADH oxidase activity of plasma membranes of rat liver (Fig. 7A). With liver plasma membranes, activity was unaffected by LY181984 even at concentrations as high as 100

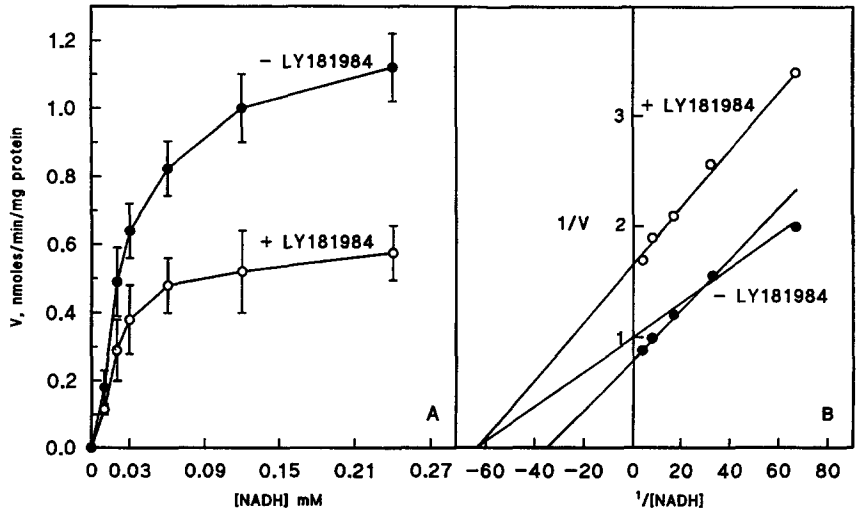

Fig. 3. The response of NADH oxidation by isolated vesicles of plasma membrane from HeLa cells frozen and thawed prior to assay. (A) As a function of NADH concentration and in the presence (open symbols) or absence (solid symbols) of 1 μM LY181984. (B) A double reciprocal analysis yielded an apparent K_m of 17 μM in the absence of LY181984 at low NADH and of 27 μM at high NADH. In the presence of LY181984, the apparent K_m was 17 μM for both low and high NADH. For each experiment duplicate determinations were averaged. Results are based on averages of three experiments ± standard deviations among experiments.

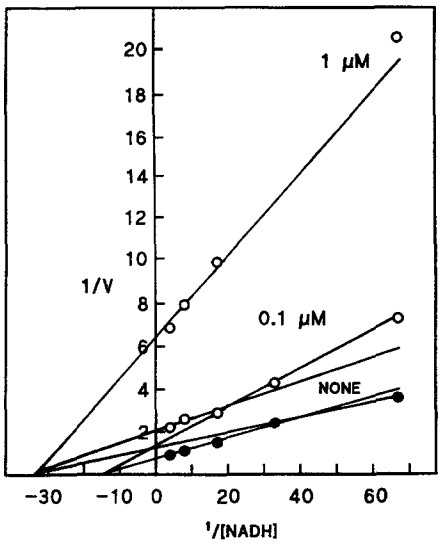

Fig. 4. Double reciprocal analysis of the response of NADH oxidation by sealed vesicles of plasma membrane from HeLa cells freshly isolated and maintained at 4°C prior to analysis comparing no LY181984, 0.1 μM LY181984 and 1 μM LY181984. In the absence of LY181984 (solid symbols), these vesicles yielded an apparent K_m of 25–30 μM at low NADH and of about 60 μM at high NADH. With 1 μM LY181984, the apparent K_m of the remaining activity was about 30 μM but the apparent V_{max} (0.15 nmol/min per mg protein) was greatly reduced from that in the absence of LY181984 (1.3 nmol/min per mg protein). Results at 0.1 μM LY181984 were intermediate. For each experiment duplicate determinations were averaged. Results are based on averages of three experiments ± standard deviations among experiments.

μM or with membranes solubilized with detergent (Fig. 7B). As with HeLa, the specific activity of the detergent-solubilized membranes (Fig. 7B) was not significantly increased over that of frozen and thawed membranes (Fig.

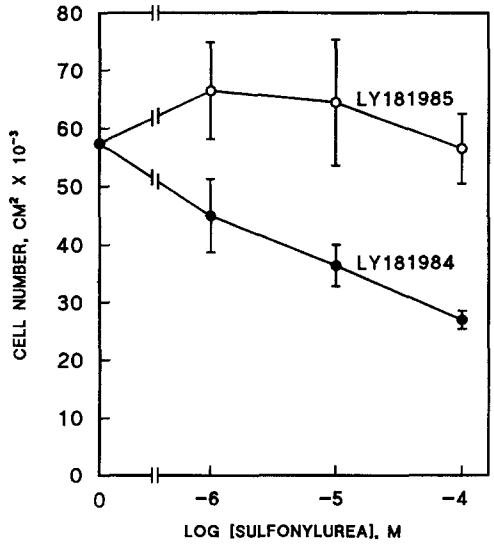

Fig. 5. Growth of HeLa cells inhibited by the antitumor active sulfonylurea LY181984 (open symbols). Several log orders more of the chemically-related but antitumor inactive LY181985 (open symbols) were required to inhibit growth. Results are from two experiments ± mean average determinations.

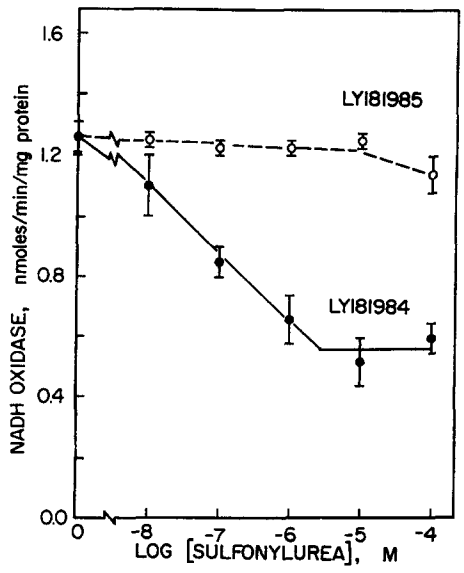

Fig. 6. Inhibition of NADH oxidase of detergent (0.1% Triton X-100)-solubilized HeLa plasma membranes by the antitumor active LY181984 (solid symbols and lines) but not by the chemically-related but antitumor inactive LY181985 (open symbols and dashed lines). For each experiment duplicate determinations were averaged. Results are averages from three experiments ± standard deviations among experiments.

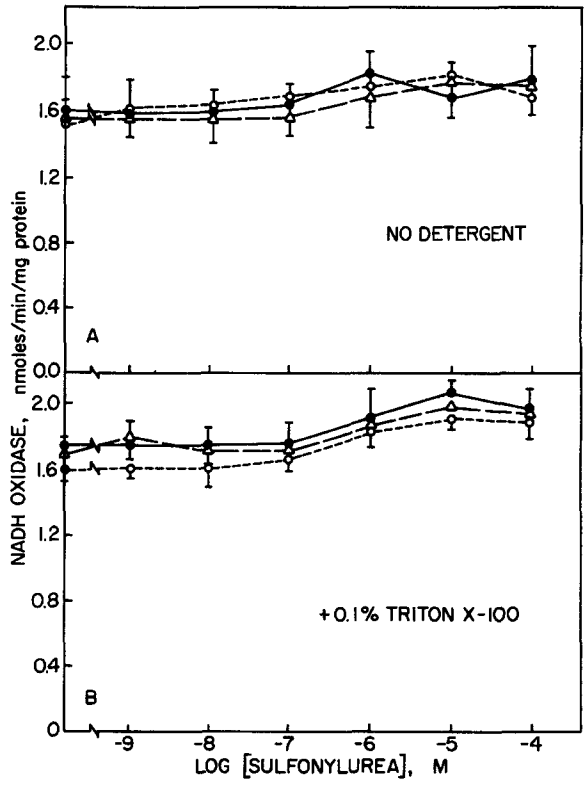

Fig. 7. Response of NADH oxidase activity of rat liver plasma membranes to increasing concentrations of LY181984 (active, solid symbols and lines), LY181985 (inactive; open circles and dotted lines) or DMSO alone (open triangles and dotted lines) in the absence (A) or presence (B) of 0.1% Triton X-100. For each experiment, duplicate determinations were averaged. Results are averages from three experiments ± standard deviations among experiments.

7A). As with liver plasma membranes, the NADH oxidase activity of normal rat kidney cells was unaffected by either 1 μM LY181984 or 1 μM LY181985 (Table 2).

4. Discussion

Plasma membranes from HeLa cells bind [^{3}H]LY181984, an active experimental sulfonylurea [10], with high affinity [1]. The binding protein has been identified tentatively as a 34 kDa component of the plasma membrane containing *N*-ethylmaleimide-reactive thiols protected against reaction with *N*-ethylmaleimide in the presence of the active sulfonylurea LY181984 [2]. By analogy with a 36 kDa sulfonylurea-inhibited NADH oxidase activity from plants [3,4], a comparable sulfonylurea-inhibited NADH oxidase of plasma membranes from sulfonylurea-responsive HeLa cells was sought.

In this report, we demonstrate an NADH oxidase activity of HeLa plasma membrane that is inhibited by the active experimental antitumor sulfonylurea, LY181984, but not by an inactive analog, LY181985. The NADH oxidase activity of liver plasma membranes which do not exhibit high affinity binding of LY181984 [1] was not inhibited by either LY181984 or LY181985. The findings implicate a 34 kDa protein with NADH oxidase activity, having a plant counterpart [3,4], as the antitumor sulfonylurea-binding protein. Inhibition by LY181984 of NADH oxidase activity of HeLa plasma membranes occurred at very low doses of drug with half maximal inhibition at about 50 nM. The concentration corresponded closely to the K_d of [^{3}H]LY181984 binding of 25 nM [1].

With predominantly intact, right side-out vesicles, the drug inhibited the NADH oxidase about 90%. However, with detergent-disrupted membranes, NADH oxidase activity was inhibited only by about 50%. This degree of inhibition was exhibited at an optimum LY181984 concentration of about 1 μM. When assays were conducted on populations of membrane vesicles that had been frozen and thawed to yield a mixture of inside-out and right side-out vesicles [24], the inhibition also was about 50%. The failure of LY181985 to inhibit more than 50% with these preparations suggests the presence in the membrane of two forms of the NADH oxidase, one inhibited by sulfonylurea, the other not.

With liver, many of the plasma membranes were isolated as sheets stabilized by junctional complexes so that both surfaces were exposed to drug and to the normally impermeant NADH substrate. Yet, the NADH oxidase activity of the plasma membrane preparations from rat liver were unresponsive to either of the sulfonylureas tested.

Results similar to those with plasma membrane vesicles from HeLa cells were obtained with plasma membrane vesicles from a plant source, soybean stems. Here, chlorsulfuron, a herbicidal sulfonylurea, inhibited both the NADH oxidase and the growth stimulated by the synthetic plant growth hormone, 2,4-D, but was without effect on the constitutive NADH oxidase activity or on growth observed in the absence of hormone [4]. Again, the results were suggestive of two forms of the NADH oxidase. One form was stimulated by the growth hormone and inhibited by the sulfonylurea. The other form was neither stimulated by the hormone nor was it inhibited by the sulfonylurea.

The basis for the inhibition when analyzed kinetically was found to be complex. With plasma membrane vesicles from HeLa cells, the sulfonylurea lowered both the K_m and V_{max} to account for the inhibition seen at saturating NADH levels. Moreover, it appeared from the kinetic analyses that the high K_m component of the NADH oxidase activity was reduced or eliminated by the addition of 1 μM LY181984 (Figs. 3 and 4). This was especially evident with frozen and thawed vesicles which contained nearly equal amounts of an activity inhibited by LY181984 and of an activity resistant to inhibition by LY181984 (Fig. 3). With isolated vesicles stored at 4°C but not frozen prior to assay, the high K_m component was less abundant and the degree of inhibition resulting from LY181984 addition was more nearly complete. Although not conclusive, the kinetic analyses not only support the overall indications of two NADH oxidase activities associated with the HeLa cell plasma membrane but further suggest that it is the high K_m, high V_{max} component of the activity that is most susceptible to inhibition by LY181984.

The plasma membrane vesicles disrupted either by detergent treatment or by freezing and thawing exhibited both sulfonylurea-inhibited and sulfonylurea-unresponsive NADH oxidase activities in nearly equal amounts on an activity basis. One possibility to explain the two forms would be an association of NADH oxidase activity with both the inner and the outer leaflets of the plasma membrane. The low K_m, low V_{max} and sulfonylurea-resistant form of the activity would represent the former. The high K_m, sulfonylurea-inhibited form of the activity would represent the latter.

The function of the plasma membrane NADH oxidase and especially of the growth factor and/or hormone-stimulated component [7] remains unknown. However, since the NADH oxidase of plasma membranes from transformed cells and tissues fails to respond to growth factors and hormones [8,9] but does respond to an antitumor sulfonylurea (this report), the oxidase, or some proteins associated with the response of the NADH oxidase to hormones and growth factors, does appear altered in response to transformation. As such the site altered in transformation might be both selective and effective as a potential target for cell surface-directed antitumor drugs.

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